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The multiverse nature of epithelial to mesenchymal transition

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Abstract

The epithelial mesenchymal transition (EMT) program is defined as a cellular transition from an epithelial to a mesenchymal state. This process occurs to provide the cell with new phenotypic assets and new skills to perform complex processes. EMT is regulated at multilayer levels, including transcriptional control of gene expression, regulation of RNA splicing, and translational/post-translational control. Although transcriptional regulation by EMT-inducing

transcription factors (EMT-TFs), including Zeb, Snail and Slug members, is generally considered the master step in this process, several evidence indicates that all these regulatory networks may have a role in the control of EMT.

There is a sort of parallelism between the biological and still unrevealed EMT complexity and the cosmological hypothesis that sustains the universe may exist as a multiverse. The presence of different EMT transition states together with the occurrence of multiple layers of regulation support the idea that EMT is just one on many out there. Is the activation of a single layer of regulation sufficient to initiate the whole EMT program? Can we postulate the activation of different EMT "dimensions"? If we think about these layers as multiple separate "universes", various scenarios can be revealed.

Keywords: Epithelial mesenchymal transition; Zeb1; plasticity

The transition of a cell from an epithelial to a mesenchymal phenotype, also named as epithelial to mesenchymal transition (EMT), has been shown in the past three decades to play critical roles in several physiological and pathological contexts, from embryogenesis to fibrosis and cancer progression. A fundamental principle of EMT is the activation of a molecular program that drives the acquisition of a mesenchymal phenotype by an epithelial cell. This program is transient, and typically ends into a mesenchymal to epithelial transition (MET). This is not an ON/OFF switch, rather an extremely flexible trans-differentiation from a state to another one and *viceversa*, recently proposed as epithelial-mesenchymal plasticity. In support of this concept, hybrid epithelial/mesenchymal phenotypes with "partially" activated EMT program have been identified in these recent years from several groups. This is well described in the context of cancer progression, where multiple tumor subpopulations are associated with different EMT states and different invasive and metastatic potentials [1].

As recently highlighted in an excellent review [2], studies *in vitro* dissected the molecular mechanisms and revealed several players with regulatory functions in EMT. These findings led to a probable over-interpretation of the results, since studies in human cancer tissues were unable to demonstrate the functional connection between EMT and metastatic spreading. Indeed, alternative modes of invasion have been described (e.g., collective cell migration, migration in cell clusters), that allow cancer cells to disseminate from the primary tumor without undergoing EMT. Therefore, controversy in the scientific community is emerged and constantly increases over time, about the real biological significance of the EMT, particularly in processes such as metastatic dissemination.

EMT arises from the dynamic interplay of different layers of regulation. Major players in the regulation of EMT are transcription factors, designated as EMT-TFs, which include Zinc finger protein SNAI1 and 2 (*SNAI1*/Snail and *SNAI2*/Slug), Zinc finger E-box-binding homeobox 1 and 2 (*ZEB1* and *ZEB2*), and Twist-related protein 1 and 2 (*TWIST1* and *TWIST2*). These factors take part to a complex network that activates a specific molecular program aimed at repressing epithelial markers (e.g., E-cadherin, *CDH1*) and activating mesenchymal markers (e.g., vimentin, *VIM*). Together with the transcriptional control of gene expression, multiple layers of regulation co-exist. For instance, extensive changes were observed at post-transcriptional and post-translational level suggesting that non-genomic alterations may have a profound impact on EMT [3]. Moreover, as a high degree of tissue specificity was described, it is clear that the presence of genetic alterations that promote or suppress the activity of EMT-TFs either directly or indirectly through mutations of upstream signaling factors may also have a fundamental role in modulating EMT [4]. Building an integrated network of regulation that accounts for interactions from all genomic and non-genomic layers is challenging. This is hampered by several factors, including the identification of key components of which each layer is composed and the generation of tools for data mining. A more complete understanding of all actors involved can yield important insights in the regulation of EMT to define the causal connections between the genotype and the observed cellular phenotype, and to pave the way to novel therapeutic strategies. If the activation or the pharmacological inhibition of one of these layers can modulate widely EMT, the perturbation of a factor presumed to exert a highly selective control of the process may have a more profound effect. The analysis of this single component may be useful to explain and predict the entire process.

As a paradigm to understand the effects of these layers on EMT regulation, we discuss a set of these alterations by focusing this review on Zeb1, a key EMT-TF that more than others plays a central role in controlling EMT activation (Figure 1). We will summarize its involvement in EMT, and will describe the genomic and non-genomic layers that control its function. The goal is to decipher the organizing principles behind its regulation operating in the temporal and spatial context of stable and hybrid EMT states.

Zeb1 and EMT

Zeb1 is a master regulator of EMT and its deregulation has been observed in a variety of cancer types and in other pathological processes [5]. Zeb1 is characterized by the presence of an amino-terminal (NZF) and a carboxy-terminal (CZF) zinc finger clusters, and a centrally located homeodomain (HD). Other protein binding domains include the Smad binding domain (SBD), the p300-P/CAF binding domain (CBD), and the CtBP interaction domain (CID) [6]. Zeb1 can repress

gene transcription by directly target to 5'-CACCT-3' E-boxes sequences located in various gene promoters, including the promoter of the master epithelial gene *CDH1* that codify for E-cadherin. Of note, the two repressor domains of Zeb1 target distinct sets of transcription factors and regulate the differentiation of specific tissues [7].

High-throughput sequencing approaches associated with overexpression/downregulation functional assays uncovered a role of Zeb1 in the regulation of multiple cellular pathways generating a long list of candidate target genes. Moreover, analysis of chromatin immunoprecipitation and sequencing (ChIP-Seq) data revealed that Zeb1 occupies a large number of DNA binding sequences that are shared with other TFs, including RREB1, RUNX2, and Smad motifs [8]. It is likely that such interaction may control Zeb1 activity as transcriptional repressor or activator depending on the diverse sets of regulatory signals and on the cellular context. These findings are supported by studies in breast cancer models, which demonstrated the role of Zeb1 as a transcriptional activator. This function is dependent on its direct interaction with specific TFs such as the Hippo pathway effector Yes-associated protein (YAP). Instead of acting on classical E-boxes, the Zeb1/YAP complex binds to particular conserved YAP/TEAD bindings sites (MCATs) located in the promoter of YAP/TAZ target genes such as *CTGF*, *CYR61* and *AXL*. The expression of this dataset is a strong predictor of reduced relapse-free and overall survival in breast tumor samples [9].

Experimental data suggest that Zeb1 is a driving force of cancer progression from precursor lesions to late-stage metastasis by providing cells with critical features, such as enhanced plasticity, altered metabolism, stemness capability, and metastatic potential [10-12]. In lung adenocarcinoma mice, heterozygous mutations of *Zeb1(+/–)* blocks benign-to-malignant transformation in *Kras*-mutated tumors, suggesting that a threshold of Zeb1 expression is required for promoting tumor initiation and progression [12]. This means that the role of Zeb1 in cancer progression is also linked to its quantitative expression.

Transcriptional regulation

The *ZEB1* gene (also known as *BZP*, *TCF8*, *AREB6*, *FECD6*, *NIL2A*, *PPCD3*, *ZFHEP*, *ZFHX1A*, *DELTAEF1*) is located on chromosome 10p11.22. Several transcript variants have been described and annotated (www.ncbi.nlm.nih.gov/gene/6935). Two alternative open reading frame (AltORF) transcripts were also identified in non-canonical +2 and +3 reading frames. These encode for two shorter proteins (UniProtKB - L0R5E9_HUMAN, and L8EAF3_HUMAN) that were recently discovered by mass spectrometry (MS/MS) [13].

ZEB1 expression is transcriptionally controlled by the coordinated action of multiple factors. Although several studies highlighted the contribution of each of these factors to the activation of

ZEB1, their global contribution remains to be defined as their activity is regulated in a context- and time-dependent manner. A direct control by the EMT-TFs Goosecoid, Snail, Slug and Twist has been reported [14]. For instance, immortalized human mammary epithelial cells (HLME) forced to overexpress Goosecoid, Snail, and Twist undergo EMT and show increased levels of Zeb1, suggesting that Zeb1 is a downstream effector of these TFs [14]. The direct transcriptional control of *ZEB1* by Slug is mediated by the binding to specific promoter elements [15]. This mechanism was observed in the melanoma cell models WM164 and WM9, where the binding of Slug to all four E-box fragments in the *ZEB1* promoter was demonstrated by ChIP. Zeb1 activation by Slug has functional consequences, as a synergic action of both TFs was observed in the regulation of EMT markers and EMT-related processes in cellular adhesion and migration. This regulation and these functional effects were specific for Slug, as Snail and Twist failed to modulate Zeb1 levels [15].

It has become clear that the effects of Snail and Slug on Zeb1 expression are strongly dependent on the tissue context. In luminal breast tumors, Snail mediates the generation of tumor initiating cells (TICs) [16]. In this model, Snail controls Zeb1 as its down-regulation but not that of Slug, reduces Zeb1 expression. Similarly, the ectopic expression of Snail in mammary epithelial cells induces its expression [16].

As demonstrated in several reports, the regulation of Zeb1 by Snail is mediated by direct and indirect mechanisms [17, 18]. In HepG2 cells, treatment with 12-O-tetradecanoyl-phorbol 13-acetate (TPA) promotes the direct localization of Snail, early growth response protein-1 (EGR-1) and SP1 to the *ZEB1* promoter. Binding of Snail occurs to a specific consensus sequence (TCACA), that is located upstream of EGR1 and SP1 binding regions. [17]. Moreover, Snail controls the transcriptional expression of *ZEB1* through the cooperation with the EMT-TFs Twist and Ets1. After stimulation with the transforming growth factor-β (TGFβ), Snail mediates the stabilization of Twist by a post-transcriptional mechanism that increases its protein stability, and Ets1 translocation to the nucleus. Knockdown of Twist was found to delay Snail protein upregulation induced by TGFβ, indicating that Twist delayed Snail protein up-regulation induced by TGFβ. Twist and Ets1 bind to distal and proximal promoter elements in the *ZEB1* promoter, thus triggering mRNA expression by TGFβ [18].

In colon cancer models, Wnt and signal transducer and activator of transcription (STAT) pathways regulate EMT by promoting Zeb1 expression [19, 20]. β-catenin/TCF4 binds directly to the Zeb1 promoter and activates its transcription [19]. Knockdown of β-catenin/TCF4 in *APC*-mutated colon cancer cells inhibits Zeb1, whereas forced translocation of β-catenin to the nucleus in *APC*-wild type colon cancer cells induces *de novo* expression of Zeb1 leading to the activation of proinvasive genes.

There is a direct correlation between Zeb1 and STAT. Zeb1 expression is significantly decreased by knockdown of STAT3 and increased by its overexpression. This is functionally linked with the presence of two STAT3 binding-sites in the *ZEB1* promoter thus supporting a direct regulation of Zeb1 expression by STAT [20]. This was further confirmed *in vivo* where simultaneous high levels of phospho-STAT and Zeb1 expression were observed by immunohistochemistry (IHC) on colon cancer tissues compared with normal colon epithelium [20].

An increased expression of Zeb1 has been associated with the activity of many other TFs including BCL6, NF-κB, SOX2, HIF1α, and FOXC2 [21-26]. This results in the activation of EMT and increased tumor aggressiveness as demonstrated by *in vitro* and *in vivo* studies [21-26]. Although the functional relationship between all these TFs in controlling Zeb1 and EMT has still to be deeply explored, the mechanism of action of each single TF is well documented. For instance, hypoxia-inducible factor 1 alpha (HIF1α) activates Zeb1 directly by binding to the hypoxia response element-3 (HRE-3) located in the *ZEB1* proximal promoter. HIF1α is also positively correlated with the levels of Zeb1 and those of other mesenchymal markers in primary and metastatic colon cancer tissue samples [24]. Forkhead box protein C2 (FOXC2), a member of the forkhead transcription factor family, promotes mesenchymal and stem cell properties [25]. In mesenchymal models, FOXC2 correlates with p38 activation, and targeting p38–FOXC2 interaction with a p38 inhibitor inhibits tumor metastasis. More in detail, it was demonstrated that p38 mediates the phosphorylation of the S367 residue of FOXC2 to induce EMT by the binding to FOXC2-binding element in the *ZEB1* promoter. Targeting of p38 or silencing of Zeb1 impairs FOXC2 signaling and regulates initiation and acquisition of stem cell features [26].

Transcriptional repressors have also a control on the transcriptional regulation of Zeb1. For instance, the retinoblastoma-associated protein 1 (Rb1) binds *ZEB1* promoter and represses thus gene expression [12, 27, 28]. The proposed model of RB tumor suppression is based on the stable formation of an E2F–Rb–histone deacetylase repressor complex [28]. This level of regulation is unique to Zeb1, as the promoters of other EMT TFs including *ZEB2* and *SNA1* can be recognized by E2F thereby influencing their expression, but through a mechanism of transcriptional regulation that does not involved and is independent from Rb. This can be interpreted as a means to regulate specifically the expression of different EMT-TFs during distinct cellular conditions.

The role of Rb1 repressor in cancer context has been described by proposing a functional connection between *RB1* gene alterations and Ras-induced Zeb1 activation [12]. In fact, the inactivation of Rb1 by mutations or deregulated cyclin-dependent kinase hyperphosphorylation creates the molecular condition for the Ras-induced expression of Zeb1. In *Kras*-mutated mouse

models of lung cancer, Zeb1 expression is required for repression of Pten and accumulation of Akt-S473, which is critical for the emergence of cancer-initiating cells [12].

Grainyhead-like-2 (GRHL2) is another transcriptional repressor of Zeb1 [29, 30]. A reciprocal negative feedback loop exists between the two transcription factors, as Zeb1 is a direct repressor of GRHL2 [28]. Ectopic expression of GRHL2 in MDA-MB-231 breast cancer cells mediates the activation of a mesenchymal-to-epithelial transition and suppresses cellular migration and invasion. By contrast, short hairpin RNA-mediated knockdown of GRHL2 in epithelial breast cancer models significantly reduced cell proliferation but did not induce EMT [31]. These data and others [32] clearly support a context-dependent role for GRHL2 and suggest that hybrid E/M and M models are more prone to undergo a complete phenotypic switch after GRHL2 knockdown.

One of the best-characterized Zeb1 repressor is Ovo-like 2 (Ovol2) [33-36]. *OVOL1/2* genes are positive regulators of epithelial terminal differentiation and directly regulate EMT genes in epidermis through the binding of specific sites on mesenchymal gene promoters [33]. Genes that are bound by Ovol2 include EMT-TFs but also genes related to cardiac differentiation or muscle function [34]. Introduction of Ovol2 to the metastatic MDA-MB-231 cells induces E-cadherin, suppresses vimentin and Zeb1 expression, and inhibits their invasive potential [34]. Thus, the expression of Ovol2 results in a complete reversion of a stable mesenchymal state. This is also confirmed in prostate cancer models, where Ovol2 over-expression promotes MET and reduces the metastatic potential of tumor cells *in vivo* [35]. In breast cancer models, Ovol2 or Zeb1 overexpression forces MCF-10A cells to acquire an epithelial or a mesenchymal phenotype, respectively [36]. The regulatory effect is bi-directional, as Zeb1 may also inhibit Ovol2 expression, regulating the transition of epithelial cells between different EMT states that are under the control of multiple inhibition loops and external activation signals [36, 37].

Epigenetic silencing

Epigenetics changes of histones, including acetylation and methylation, were observed in epithelial and mesenchymal models and associated with the activation or repression of EMT markers [38]. Site-specific histone modifications exert a major control on *ZEB1* [39]. An increased in *ZEB1* expression was observed in basal breast cancer models that show high metastatic potential and high expression of mesenchymal markers. These cells show high levels of CD44 and display a chromatin methylation pattern that indicates an active transcription at the *ZEB1* promoter, as revealed by the presence of both trimethylation of lysine 4 of the histone H3 subunit (H3K4me3) and dimethylation of lysine 79 of the same subunit (H3K79me2) marks. In contrast, $CD44^{low}$ cells with luminal and basal features present a different bivalent chromatin methylation patterns as determined by the

presence of H3K4me3 and H3K27me3, respectively. After stimulation with TGFβ only basal $CD44^{low}$ cells are able to transform into a $CD44^{high}$ phenotype and increase *ZEB1* expression. This is obtained by a transition from a bivalent chromatin status to an active chromatin state through a significant decrease of the H3K27me3 mark [39]. These histone modifications are regulated by specific histone methyltransferases (HTMs) and histone demethylases (HDMs) that regulate the EMT status targeting Zeb1 and other EMT-TFs [40].

Post-transcriptional regulation

Several mechanisms regulate EMT at post-transcriptional level by controlling RNA splicing and stability. For example, an EMT-associated alternative splicing (AS) signature was identified in breast cancer cell models and primary tumors and associated with different classes of splicing factors including RBFOX, MBNL, CELF, heterogeneous nuclear ribonucleo-protein (hnRNP), or epithelial splicing regulatory proteins (ESRPs) [41]. Silencing of these factors can in part regulate EMT and *viceversa*. Indeed, changes in the expression of EMT-TFs including Zeb1 have a widespread impact on AS during EMT, through the regulation of ESPRs, RBM47 and QKI factors and the establishment of a specific epithelial or mesenchymal splicing programme [42]. The functional significance for some of these AS isoforms has been demonstrated. The ectopic expression of Zeb1 in HLMER cells induces the expression of RBFOX1 and QKI RNA binding proteins that act by promoting the acquisition of a mesenchymal state through the regulation of the actin binding protein filamin B (FLNB) [43]. Importantly, such alterations in the AS pathway are critical for tumor cells to generate an alternative protein isoform of FLNB able to induce EMT by releasing the TF FOXC1 from an inhibitory complex [43].

Mechanisms for generating heterogeneity can also act on the regulation of protein turnover through the stabilization of the mRNA 3'-untranslated region (3'-UTR). Diverse RNA binding proteins (RBPs) are involved in the regulation of *ZEB1* mRNA stability including the AU-binding factor 1 (AUF1), also known as hnRNPD, and the polypyrimidine tract-binding protein 3 (PTBP3). Both proteins promote the stabilization of Zeb1 transcript [44-46]. The expression and function of RBPs can be regulated in several ways with a consequent effect on *ZEB1* mRNA expression and EMT induction. For instance, AUF1 is a target of beclin1 and of the two tumor suppressor miRNAs miR-141 and miR-146b-5p [44, 46].

Altered miRNA(miR) expression is a characteristic of EMT [47], and a specific miR signature is associated with the different epithelial and mesenchymal features of cancer models [48].

Several miRNA members have emerged as negative regulators of EMT process by directly targeting of *ZEB1* mRNA. Among them, the best characterized are the members of the miR-200

family (miR-200a/b/c, miR-141 and miR-429) [49]. Recent miRNA sequencing strategies led to discover other actors, including the miR-1199-5p, that contribute to the regulation of EMT both *in vitro* and *in vivo* [50]. These miRNAs destabilize *ZEB1* transcript after the binding to 3'-UTR sequence-specific regions, resulting in a reduced expression of Zeb1.

In tumor cells, miR-200 family and Zeb1 operate in a double-negative feedback loop [51] that is under the control of multiple factors including external stimuli such as TGFβ [52, 53]. In detail, two different models of reciprocal regulation were proposed and experimentally validated. In the cascading bistable switches, miR-34 and miR-200 function together as a bistable switch to control EMT [52]. Low levels of these miRNAs and high levels of EMT-TFs Snail and Zeb1 regulate the existence of an epithelial, hybrid or mesenchymal state [52]. In the three stable states, the Zeb/miR-200 loop allows for the existence of three phenotypes characterized by an epithelial (high miR-200, low Zeb), a mesenchymal (low miR-200, high Zeb) and a hybrid (medium miR-200, medium Zeb) phenotype [53].

Perturbations of the miR-200/Zeb1 network and alteration of the EMT status can occur in response to external stimuli as observed with TGFβ or through the regulation by other miRNA. For instance, miR-22 promotes EMT and stemness in breast cancer models by repressing miR-200a and miR-200c expression leading to Zeb1 up-regulation. By the direct targeting of the Ten eleven translocation (TET) family of methylcytosine dioxygenases, miR-22 controls the methylation status of the miR-200 promoter and its subsequent expression and EMT suppression. By the modulation of miR-200/Zeb1 network, miR22 appears to make an important contribution to cancer metastasis as its overexpression significantly correlates with poor clinical outcomes in patients [54].

Mutations or modifications in the 3'-UTR of *ZEB1* that alter the binding of miRNA may represent a mechanism to avoid miRNA regulation. For instance, a progressive shortening of the *ZEB1* transcript was observed in pancreatic ductal adenocarcinoma (PDAC) cells exposed to the drug gemcitabine [55]. The treatment leads to shortening of the 3'-UTR region by the selection of alternative polyadenylation signals (PAS). This causes the selective deletion of binding sites for repressive miRNAs [55].

Long non-coding RNAs (lncRNAs) were recently identified as potential regulators of EMT [56, 57]. It is now widely understood that lncRNAs are finely regulated and required to support tumor growth and metastasis *in vivo*. Specifically, a different lncRNAs signature is activated when EMT is induced through external EMT-stimuli as TGFβ or by the forced expression of EMT-TFs [56, 57]. Major mechanisms of regulation of gene expression involve the interaction with the epigenetic silencing complex polycomb repressive complex 2 (PRC2), and the ability of lncRNA to act as competing endogenous-RNAs (ceRNAs) for EMT-regulatory miRNAs [58].

Studies have demonstrated that some well-characterized lncRNAs can modulate Zeb1 levels by acting as ceRNAs and impairing miRNA activity [59-63]. For example, lncRNA XIST (X-inactive specific transcript) and highly up-regulated in liver cancer (HULC) can indirectly regulate Zeb1 by targeting miR-200b-3p [59, 60]. LncRNA promotes epithelial-mesenchymal transition (lncRNA-PE) represses the expression of two members of the miR220 family, miR-200a/b, thereby promoting EMT by Zeb1 expression [59]. Similarly, another work described the downregulation of miR-574-5p by linc-ZNF469-3 in triple negative tumors [62]. Further experiments showed that HOTAIR enhances EMT in hepatocellular carcinoma (HCC) cells via sponging miR-23b-3p from Zeb1 [63]. In all of these cases, cancer growth and metastatic potential are significantly associated with the expression of these lncRNAs through their regulation of Zeb1.

The lncRNA Zeb1 antisense 1 (ZEB1-AS1), which derives from the promoter region of *ZEB1*, can contribute to activation of the EMT programme by regulating Zeb1 levels [64, 65]. In colorectal cancer (CRC), ZEB1-AS1 functions as a molecular sponge for the miR-101 that directly targets and inhibits Zeb1 expression [64]. Higher levels of ZEB1-AS1 were observed in CRC tissues compared to normal samples and correlated with higher histological grade and advanced tumor stage. Functionally, ZEB1-AS1 inhibition combined with miR-101 overexpression significantly inhibited CRC cell proliferation and migration [64]. Multiple mechanisms of action have been proposed for ZEB1-AS1 regulation of Zeb1. For example, in prostate cancer models, ZEB1-AS1 binds to the H3K4 methyltransferase MLL1 and promotes H3K4me3 histone modification in *ZEB1* promoter by changing the chromatin status from an inactive to an active state [65].

Translational and post-translational regulation

At the translational level, EMT has benefited from the application of mass spectrometry studies that allowed a deep characterization of a large number of cellular processes, or pathways modified after the activation of EMT or after the knockdown of selected EMT markers [66-69]. These studies provided a large catalogue of proteins that are over- or under-expressed or post-translationally modified and suggested that modifications at the proteome level are critical for EMT induction.

A variety of post-translational modifications (PTMs) have been implicated in the modulation of Zeb1 activity, as well as of many other EMT-TFs. To date, the known PTMs of Zeb1 include phosphorylation, ubiquitination, and SUMOylation (www.phosphosite.org). As an example, the role of phosphorylation in regulating Zeb1 has long been recognized [70]. These modifications either positively or negatively regulate Zeb1 activity with an effect on protein stability or nuclear localization. For instance, the phosphorylation status of Zeb1 affects some basic protein processes and localization [70]. In detail, an increased DNA-binding activity was observed when native Zeb1

was de-phosphorylated. Multiple signaling pathways regulate Zeb1 phosphorylation, and treatment of tumor cells with specific inhibitors of MEK/ERK, PKC, or PI3K kinases results in increased Zeb1 binding to DNA [71]. A further challenge is the fact that different phosphorylation sites on Zeb1 amino acid sequence can account each for a different role on Zeb1 activity. In breast cancer cells, in response to γ-ionizing radiation, resistant tumor cells activate the serine/threonine protein kinase ATM that phosphorylates and stabilizes Zeb1 through phosphorylating its serine 585. Zeb1 in turn interacts with and promotes the activity of ubiquitin-specific-processing protease 7 (USP7), which deubiquitinates and stabilizes that serine/threonine kinase checkpoint kinase 1 (CHK1) thus promoting DNA repair and resistance to radiation [72].

Ubiquitination controls the stability of a variety of EMT-TFs contributing to the activation or repression of EMT [73]. A regulation of Zeb1 levels by ubiquitination has been described [74-76]. For instance, an atypical E3 ubiquitin ligase Skp1-Pam-Fbxo45 (SPF^{Fbxo45}) directly mediates the degradation of different EMT-TFs including Zeb1, Zeb2, Snail, Slug, and Twist [74]. In breast cancer models, Siah proteins, a family of E3 ubiquitin ligases, contribute to EMT by regulating Zeb1. Siah protein expression is decreased upon treatment with TGFβ, while its knockdown up-regulates mesenchymal genes [75]. Similarly, the parkinson protein 2 (PARK2) E3 ubiquitin protein ligase acts as a metastasis suppressor in glioblastoma multiforme (GBM) progression by targeting Zeb1 [76].

Deubiquitinating enzymes can promote Zeb1 deubiquitination and stabilization [77]. The ubiquitin-specific protease-51 (USP51) binds, deubiquitinates, and stabilizes Zeb1. USP51 is overexpressed in human breast tumors and correlates with shorter overall survival. *In vitro,* ectopic expression of USP51 up-regulates Zeb1 and the mesenchymal markers N-cadherin and Vimentin [77].

The protein caspase-8-associated protein 2 (CASP8AP2 or FLASH) protects Zeb1 from proteasomal degradation induced by SIAH1 and FBXO45 and increases its protein turnover [78]. The loss of FLASH decreases Zeb1 levels but not the mRNA expression of *ZEB1*, with a consequent increase in *CDH1* expression. FLASH-depleted PANC-1 cells failed to up-regulate Zeb1 in response to TGB-β treatment and retain an epithelial phenotype [78]. This means that prevention of Zeb1 degradation by the proteasome was necessary for the activation of EMT after the activation of signaling processes.

Impact of Zeb1 on cancer prognosis

Although Zeb1 is considered as a master regulator of EMT, its impact on diagnosis of human cancer prognosis remains highly dependent on the tumor type. EMT and metastasis are generally considered late events in tumorigenesis, although EMT and cancer dissemination has been

described very early in tumor progression of pancreatic cells and in parallel to tumor onset at the primary site [79]. Overall, this suggests a key role for EMT in early steps of the malignant progression by inducing chemo-resistance and CSCs phenotypes [80], or by preventing oncogene-induced cellular stress [81].

A recent study showed that high expression of Zeb1 is associated with shorter overall survival within the stage IA lung cancers that do not invade local lymph nodes; this indicates that detection of $Zeb1^+$ cells can predict for metastatic disease [9]. Using genetically engineered mouse models (GEMMs) of pancreatic cancers, it was demonstrated that Zeb1 ablation is associated with tumors of the "classical subtype", which has the best clinical prognosis [10], while absence of either Twist1 or Snai1 did not affect the capacity of pancreatic cancer cells in GEMMs to local or distant (lung and liver) spreading [80].

In breast cancer, EMT-TFs expression and association with prognosis remain controversial. A recent meta-analysis of the literature data showed that the expression of Snail, Slug and Twist is associated with an increased risk of developing metastasis and poor survival. On the other hand, the risk of poor prognosis associated with Zeb1 expression was substantially lower than those of the other EMT-TFs [82].

Do we live in an EMT universe or multiverse?

EMT is a complex process coordinated at multiple levels, including epigenetic, transcriptional and post-transcriptional regulation. Dynamic and precise regulation of these processes is essential to drive the transition between epithelial and mesenchymal phenotypes. Moreover, recent studies in cancer biology support the view that, rather than two simple states, hybrid states with a different clinical relevance can exist [1, 83-86]. The existence of these intermediate, plastic states led to a completely new global view of EMT, as we can no more consider this as a black or white process but as a more complex biological problem in rapid evolution, setting the hypothesis of finite or infinite possible states that are determined by the quantitative and qualitative different expression of EMT markers. In fact, if we want to derive the weight of each EMT marker in the determination of a specific hybrid state, we should focus on its qualitative status (switched ON/OFF), as well as on its quantitative expression level. If we place this in relation to the high number of genes that are modified during EMT [87], we can assume that infinity or tending to infinity EMT status may exist. This also made a number of new questions only in part validated experimentally. How these transition states can be generated? Do they emerge by chance or specific molecular networks regulate them? Do they share a common progenitor? Can they co-exist simultaneously or these are

regulated in time and space-dependent manner? Which is their biological role during cancer onset and progression? Do they act in cooperation?

This greater variability was observed *in vivo*, where a wide range of expression of EMT markers led to the classification of human tumors into several groups with different EMT scores [87]. Some types including colorectal or gastric tumors were characterized by a higher epithelial score and expression of epithelial markers, while others including osteosarcoma or glioblastoma showed an opposite trend with an enrichment of mesenchymal markers [88]. As mentioned in several studies, the presence of different EMT states may have therapeutic implications. For instance, co-expression of epithelial and mesenchymal genes that functionally predicts an interaction between epithelial and mesenchymal cells is associated with poor clinical outcomes in breast and ovarian cancer tissues [85, 86]. Additionally, the existence of distinct epithelial and mesenchymal states has also been reported in the context of breast cancer stem cells, where two distinct populations of CSCs were identified. The plasticity of CSCs to transit between a mesenchymal-like and an epithelial-like state is critical to regulate the formation of metastasis at distant sites [89]. This is consistent with what is observed during embryonic development, where the presence of hybrid EMT states that are positively correlated with stemness properties has also been reported [90]. As a consequence of this variability, more precise approaches for the analysis of tumor cells are required, including single-cell RNA-sequencing (scRNA-seq) methods. Recent insights into head and neck squamous cell carcinoma (HNSCC) using scRNA-seq of single tumor cells, led to the identification *in vivo* of a metastable EMT program localized to the leading edge of primary tumors and associated with metastasis [91].

Which are the molecular determinants of these different transition states? If they act in a hierarchical way, can we have a control on different EMT states by blocking the expression of selected determinants or by interfering with their regulatory networks? As described, transcriptional control mechanisms through different EMT-TFs have a role of regulation, and in combination with specific molecular circuits can modulate the acquisition of stable and intermediate EMT states [92, 93]. Among these TFs, Zeb1 governs the terminal differentiation into the mesenchymal state in epithelial tumor progression and the acquisition of CSCs properties. This is consistent with the function of Zeb1 as regulator of cell differentiation in different normal tissues, including nervous systems, adipose tissue, and muscle [94-96]. This plasticity can be easily explained considering the multiple mechanisms that regulate Zeb1 expression (Figure 2). In the examples reported in this review, various factors cooperate each other in well-defined molecular networks, are affected by the stimuli from the surrounding microenvironment, and are modulated by the genetic background in a tissue-dependent manner. Acting on Zeb1 by increasing its level of expression through the

modulation of these layers of regulation triggers the differentiation into a stable EMT state, while its inhibition promotes the transition towards other stable or metastable states. These transitions can occur stochastically [97] or guided by the action of chemical stimuli including drugs [98]. The fact that tumor cells show plasticity in the setting of metastasis or response to therapy highlights the clinical importance of this process. As such, a deeper understanding of these mechanisms of regulation may pave the way to the design of new therapeutic strategies to target EMT plasticity through the integration of these multiple layers of data into new circuits of regulation. At the present, the biological relevance of some of these layers as well as their relevance in the context of EMT remains to be confirmed. For instance, the presence of two alternative Zeb1 proteins may suggest new hypotheses about the possible functional relationship between these alternative transcripts and the protein codified by the main coding sequence [99]. Interestingly, as multiple examples of AltORFs have been identified in the main sequence of other EMT markers [100], it is likely that this mechanism of regulation may have a role in controlling EMT reprogramming.

According to this view of high cell plasticity and dynamism, a drug combination strategy aimed at targeting these hybrid states may have significant effects compared to a single therapeutic agent. In fact, recent studies indicate that the targeting of two or more elements of the TGFβ network is more effective to suppress EMT than targeting a single element [101].

In summary, *in vivo* evidence that support the presence of multiple hybrid states of EMT are increasing. However, there is increasing debate about the clinical relevance of stable and metastable states, with recent studies supporting the functional role of hybrid states. In this scenario, while targeting Zeb1 has demonstrated to be valuable *in vitro*, it remains to be confirmed *in vivo* where the inactivation of such factor may probably promote the transition to other EMT states with increased malignant phenotype.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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Figure legend

Figure 1. The Ingenuity Pathway Analysis (IPA) tool was used to generate a graphical representation of the canonical pathway "Regulation of the Epithelial-Mesenchymal Transition Pathway". Violet circles indicate Zeb1. A) Zeb1 orchestrates the activation of the EMT program acting as key effector TF for the differentiation of epithelial cells. Zeb1 together with other EMT-TFs drives the activation of a mesenchymal program with a direct functional effect on multiple biological processes including invasion, migration and metastasis. B) Zeb1 is activated by multiple signaling stimuli from the microenvironment including the TGFβ and EGF canonical pathways. The symbols used to represent molecules were illustrated in the legend.

Figure 2. Key regulatory mechanisms controlling Zeb1 expression. The activation of one or more of these layers of regulation such as transcription, epigenetics, translation and protein degradation acting on Zeb1 can exert a significant effect on the EMT/MET differentiation status.

A) The binding of various EMT-TFs at specific regions of Zeb1 promoter regulates gene expression at transcriptional level. EMT-TFs can act directly or through the binding of transcriptional cofactors.

B) The methylation status of Zeb1 promoter control gene expression. Compared to cell models that are in a stable epithelial state, the ZEB1 promoter of metastable cells is maintained in a bivalent chromatin configuration and prone to react to the external stimuli.

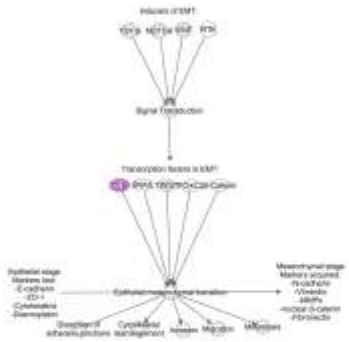
C) Two AltORFs overlapping the main cDNA coding sequence but encoded with a different reading frame give rise to different proteins, and these were identified by MS/MS.

D) An extension of ternary chimera switch (TCS) model shows the different action of miR-22, miR-1199-5p and Zeb1-AS1 on Zeb1 transcript regulation.

E) Mechanisms of regulation that stabilizes Zeb1 transcript acting on its 3'-UTR region were described. Specific trans-acting factors (proteins and miRNAs) have a strong impact on this mechanism of regulation.

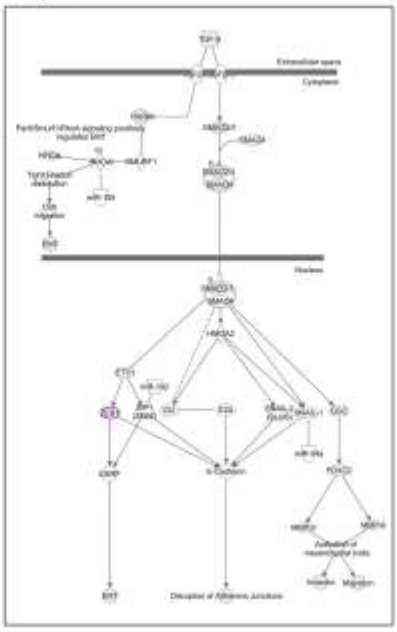
References for examples: transcriptional control [15, 17, 18], chromatin status [39], AltORFs [13], miR and lncRNA regulation [50, 54, 64, 65], 3'-UTR regulation [44, 46].

A



B

TGF-β and EMT



Receptor Tyrosine Kinases and EMT